AD_____

Award Number:

W81XWH-09-2-0081

TITLE:

EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

PRINCIPAL INVESTIGATOR:

Thomas S. Kilduff, Ph.D.

CONTRACTING ORGANIZATION:

SRI International
Menlo Park, CA 94025-3493

REPORT DATE:

September 2010

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 01-09-2010 | Annual | 1 Sep 2009 - 31 Aug 2010 |

**4. TITLE AND SUBTITLE**
EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-09-2-0081

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Kilduff, Thomas S, Ph.D.

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

SRI International

Menlo Park, CA 94025-3493

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

During Year 1, progress was made on establishment of the infrastructure and baseline parameters to facilitate the proposed studies. Construction was initiated on a 535 s.f. lab suite within the Animal Facility that will provide an optimal environment for execution of the proposed in vivo studies; we expect to occupy this laboratory next week. An Analytical Neurochemistry Facility was established in LB212 and 4 HPLCs were purchased, installed, calibrated and the minimal detection levels for measurement of several neurotransmitters were determined. Over 25 g of the test compound, the hypocretin receptor antagonist almorexant (ALM), was synthesized, resulting in achievement of the first milestone. The effects of 3 concentrations of ALM on sleep/wake, locomotor activity and body temperature were determined. The behavioral performance studies were initiated in a temporary location. We are now well-positioned to rigorously test the hypothesis in Years 2-4 that ALM produces fewer functional impairments than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems.

**15. SUBJECT TERMS**
Sleep, performance, drug, neurotransmitter, hypocretin, orexin, benzodiazepine, zolpidem, neurochemistry, microdialysis

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 28 | **19b. TELEPHONE NUMBER** *(include area code)* |

## Table of Contents

**PROGRESS REPORT**
"EFFECT OF A HYPOCRETIN/OREXIN
ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE"
USAMRAA Grant W81XWH-09-2-0081
CDMRP Log No. DR080789P1
Thomas S. Kilduff, Ph.D., Principal Investigator

## INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces fewer functional impairments than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component will establish if ALM is superior to ZOL in neurocognitive tests, the animal studies will compare the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

## BODY

As indicated in a 21 Jul 2010 email from the PI, Dr. Thomas Kilduff, to Dr. Kimberly del Carmen, Health Sciences Grants Manager for the Congressionally Directed Medical Research Programs, SRI International has undertaken construction of a new lab for the experiments to be conducted under DR080789P1 (USAMRAA Grant W81XWH-09-2-0080), the "Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance". This construction was necessitated because of the type of experiments that were proposed in DR080789P1. Our laboratory for collecting microdialysis samples from rodent brain has been located next to the cage-washing room in the Animal Facility for several years. Although this location was adequate for most microdialysis studies, the location was problematic for the studies proposed in the "Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance" since we planned to record sleep/wake in conjunction with obtaining microdialysis samples. This location is a "high traffic" area as cages are moved down the hall and into the washer and up the hall once they have been washed.

Consequently, upon funding of DR080789P1 (USAMRAA Grant W81XWH-09-2-0080), we began planning the construction of a new 535 sq foot laboratory suite in which to conduct the proposed EEG/EMG, behavioral performance and microdialysis studies (see Appendix 1). We anticipated that this would be a minor construction project that would last 6-8 weeks. Unfortunately, the project exceeded a cost threshold which necessitated that the City of Menlo Park to approve the plans, causing the first set of delays. The City assessed our project in the context of the overall facilities in which the laboratory was located and determined that the building as a whole was not compliant with current Americans with Disabilities Act (ADA) regulations. Accordingly, our project triggered a requirement that wheelchair access be provided to the building and that wheelchair-accessible bathrooms be installed. Since the scope of these City-imposed requirements greatly exceeded the scope of our original project, there were further

delays as plans were drawn up and the details of the now-expanded project were negotiated internally. Once construction began, not only was the scope of the construction project larger, but it had to be conducted in a manner that was minimally disruptive to ongoing experiments that were being conducted in the Animal Facility. Thus, construction has been limited to 3 days per week.

Construction on this project began on 19 May 2010, is currently proceeding apace, and is projected to be completed by 03 Aug 2010. We are looking forward to occupying this new laboratory in early August and accelerating progress on the "Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance" in this new lab. However, as indicated in the Progress Report below, we have fallen behind on the goals that we established for Year 1 for DR080789P1 (USAMRAA Grant W81XWH-09-2-0080) in the SOW. Although there has been some progress on each of the Tasks, we will achieve fewer of the Milestones in Year 1 than we originally projected.

**Task 2.** *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*
2a. Assessment of Almorexant effects on spatial reference memory in rats (months 1 to 12).
2b. Assessment of Almorexant effects on spatial working memory in rats (months 1 to 12).
2c. Assessment of Almorexant effects on psychomotor vigilance in rats (months 13 to 24).
2d. Synthesis of ALM (months 1-4).

Progress: Task 2d was added to SOW in August 2009 in case problems arose with respect to the donation of ALM that was expected from Actelion Pharmaceuticals Ltd. Logically, however, ALM had to be available before any studies could occur. Thus, Task 2d was the first Task completed. As indicated in the Certificate of Analysis (Appendix 2), **this milestone has been achieved** as 26.05 g of >99% pure almorexant was delivered by the SRI Medicinal Chemistry Laboratory on 31 Mar 2010.

As indicated above, we have been limited in our ability to conduct the studies proposed as Tasks 2a and 2b in the SOW due to the ongoing construction. In May 2010, a small animal experimental room unexpectedly became available for our use on a temporary basis due to the departure of an investigator from SRI. This room was just adequate in size to house the Morris water maze to be used in Aims 2a and 2b. Therefore, we have set up the water maze and video tracking system in this room on a temporary basis and have initiated the studies for Aim 2a and are current collecting data.

Prior to undertaking any of the proposed studies in Tasks 2-5, we had to be certain of the doses of ALM and ZOL to be used for treatment of the animals in these studies. We report here on the results of a study of the effects of ALM and ZOL on sleep and wakefulness undertaken in collaboration with colleagues at F. Hoffman la Roche. Although these experiments were initiated prior to funding of DR080789P1 (USAMRAA Grant W81XWH-09-2-0080), the analysis has only been completed within the past year and the results are directly relevant to the "Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance". Male Sprague-Dawley rats (300±25 g) used in this study were from Charles River (Wilmington, MA) and were housed in a temperature-controlled recording room under a 12 h light/12 h dark cycle (lights off at 05:00) with food and water available *ad libitum*. Room temperature (24±2$^{o}$C), humidity (50±20% relative humidity), and lighting conditions were monitored continuously via computer. Animals were inspected daily in accordance with AAALAC and SRI guidelines.

Experimental design. Eight male Sprague-Dawley rats (300±25 g; Charles River, Wilmington, MA) were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of electroencephalograph (EEG), electromyograph (EMG), core body temperature ($T_{core}$), and LMA via telemetry as previously described previously (Morairty et al., 2008). Data were recorded using DQ ART 3.1 software (Data Sciences Inc., St Paul, MN). Animals were acclimated to the handling procedures and were given two separate 1 ml doses of vehicle, one 7 d and the other 3 d before the first experimental day. Following completion of data collection, expert scorers determined states of sleep and wakefulness in 10 s epochs by examining the recordings visually using Neuroscore software (Data Sciences Inc., St Paul, MN). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. The EEG and EMG data were scored for waking (W), rapid eye movement sleep (REM), and non-REM (NR). $T_{core}$ and LMA (counts per minute) were analyzed as hourly means.

A repeated measures design was employed in which each rat received five separate dosings. The dosing conditions included almorexant at three concentrations (10–100 mg/kg), ZOL (10 mg/kg) and a vehicle control (HPMC). All dosings were administered ip at a volume of 2 ml/kg. A minimum of 3 d elapsed between doses. Dosing occurred during the middle of the rats' normal active period during the start of Zeitgeber hour 19 (ZT19) and was typically completed within 10 min. Animals were continuously recorded for 6 h prior to dosing and for 18 h following dosing.

Data analyses. EEG and EMG data, scored in 10 s epochs as described above, were analyzed as time spent in each state (W, REM, and NR) per hour. Latency to NR onset for each rat was calculated from the time of drug injection to the first six continuous 10 s epochs scored as NR. Latency to REM onset for each rat was calculated from the time of drug injection to the first three continuous 10 s epochs scored as REM. Cumulative time spent in W, NR, and REM, as well as the REM:NR ratios, were calculated for 6 h following drug administration. To determine whether any of the pharmacological treatments affected the consolidation of behavioral states, the duration and number of bouts for each state were calculated in hourly bins. A "bout" consisted of a minimum of two consecutive 10 s epochs of a given state and was terminated by the occurrence of a single epoch of a different state. The EEG spectra during NR sleep were analyzed offline using the fast Fourier transform algorithm in Neuroscore (Data Sciences Inc., St Paul, MN) on all epochs without a visually detectable artifact. EEG delta power (1–4 Hz) within NR (NRD) was then calculated in hourly bins. $T_{core}$ and LMA (counts per minute) were analyzed as mean values per hour (hourly means). Relative $T_{core}$ was calculated as the difference in $T_{core}$ from the 24 h average during the vehicle condition.

Statistics. The records were analyzed in 6 h time blocks (i.e., first half of the dark period, second half of the dark period, first half of the subsequent light period, and second half of the light period) since drug administration occurred 6 h into the recording period. Latency to NR and REM, REM:NR ratios, and cumulative state data were analyzed using one-way repeated-measures analysis of variance (ANOVA); all other data were analyzed using two-way repeated-measures ANOVA. When ANOVA indicated statistical significance, paired two-tailed *t*-tests were performed for post hoc analysis.

Results. Almorexant at 30 and 100 mg/kg reduced NR latency while only the 30 mg/kg concentration decreased latency to REM sleep (Figure 1). ZOL produced the expected decrease in NR latency.

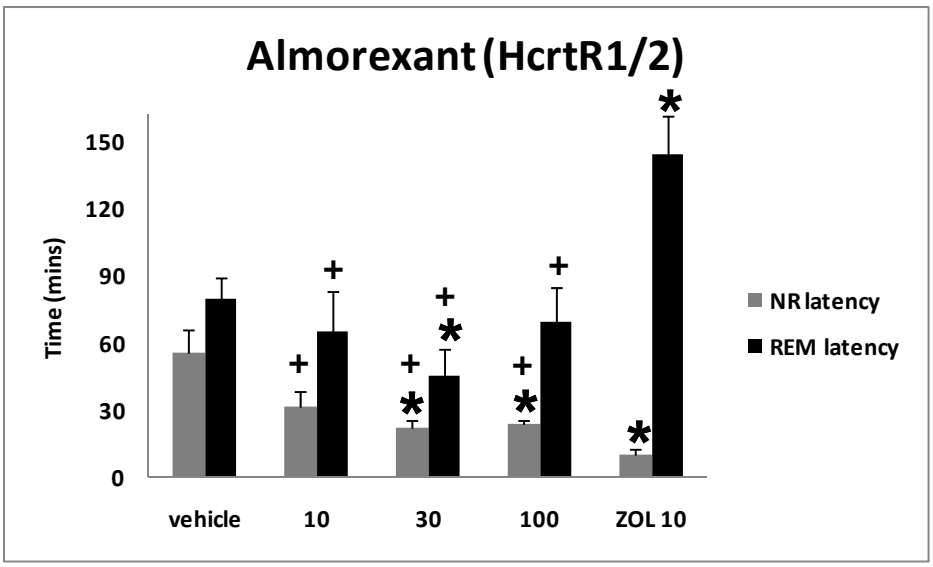



**Figure 1.** Latency to the onset of NR and REM sleep following administration of almorexant as compared to zolpidem (ZOL). *=significantly different from vehicle (P<0.05); +=significantly different from ZOL (P<0.05) (One-way repeated measures ANOVA followed by paired two-tail *t-tests* test; n=8 per group). Data represent the mean ± SEM.

As illustrated in Figure 2, almorexant (30 and 100 mg/kg) resulted in increased cumulative NR for 2, 4 and 6 h following administration (F=13.010, P<0.0001; F=17.771, P<0.0001; and F=16.179, P<0.0001, respectively). Cumulative REM also increased for the first 2 h following almorexant at 30 mg/kg (F=5.418, P=0.0023) and for the 6 h period following the 100 mg/kg dose (Figure 2; F=8.535, P<0.0001). ZOL increased cumulative NR and decreased cumulative REM. Consequently, whereas ZOL suppressed the REM:NR ratio, ALM did not.

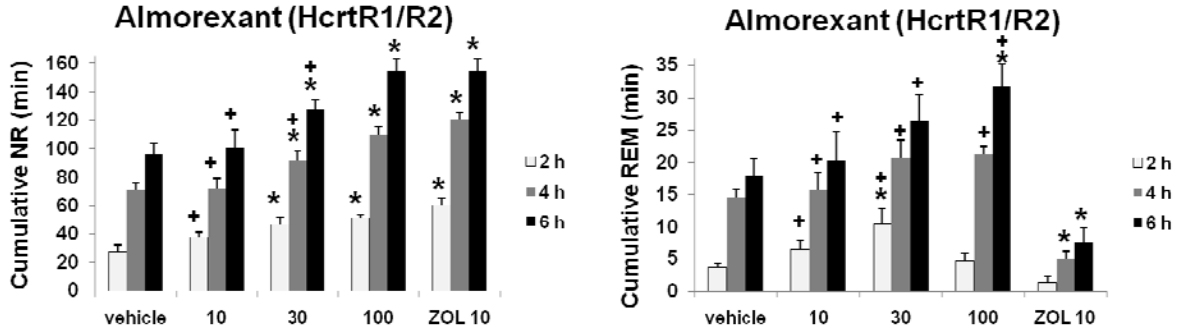



**Figure 2.** Cumulative time in NR and REM sleep over the first 2, 4 and 6 h following drug administration. **Left:** cumulative time spent in NR sleep following almorexant compared to zolpidem (ZOL). **Right:** cumulative time spent in REM sleep for the same drug treatments. *, significantly different from vehicle; +, significantly different from ZOL.

Although ZOL has significant effects on NRD, ALM did not (Figure 3).

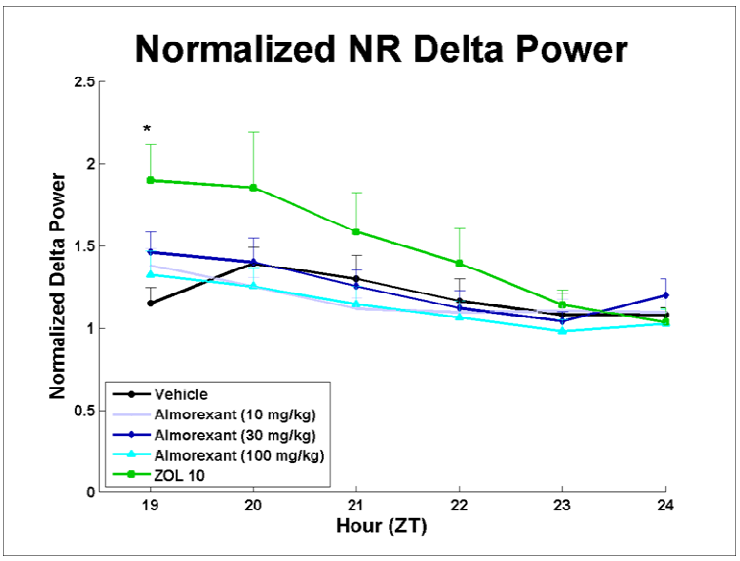



**Figure 3.** Hourly delta power normalized to the 24 h average vehicle control. 3 concentrations of almorexant vs. ZOL and vehicle.

Both LMA and $T_{core}$ underwent dose-dependent decreases after drug treatment (Figure 4). No differences in LMA during the subsequent light period were found. Condition effects for $T_{core}$ were found. The highest concentration decreased $T_{core}$ across the 6 h period following administration (F=7.315, P=0.00036). ZOL administration resulted in the largest declines in $T_{core}$, which was followed by a sustained rebound increase in $T_{core}$ during the subsequent light period.

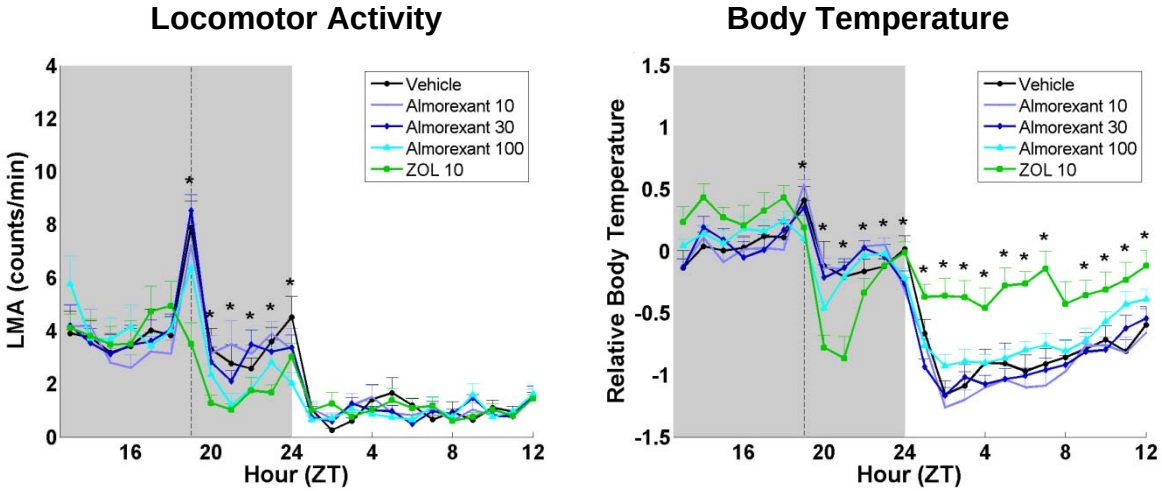



**Figure 4.** Average hourly LMA and relative $T_{core}$ for 6 h prior to and 18 h after injections. Shaded area indicates dark phase; the dashed line in each panel indicates the first h following dosing. **Left:** The average hourly LMA for 3 concentrations of almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment (F=7.31, P=0.00036) and for treatment by time (F=2.38, P=0.0018). For treatment by time: At ZT19, ZOL<all other conditions. At ZT20, ZOL<almorexant at 10 mg/kg and vehicle. At ZT21, ZOL<almorexant at 10 and 30 mg/kg and vehicle. At ZT22, ZOL<almorexant at 30 mg/kg and vehicle. At ZT23, ZOL<almorexant at 10

and 30 mg/kg and vehicle. At ZT24, almorexant at 100 mg/kg and ZOL<vehicle. **Right:** The average hourly $T_{core}$ for 3 concentrations of almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment (F=7.55, P=0.00029) and for treatment by time (F=3.97, P<0.00001). ANOVA for ZT1-ZT6 is significant for treatment (F=15.75, P<0.00001) and for treatment by time (F=1.95, P=0.0134). ANOVA for ZT7-ZT12 is significant for treatment (F=7.92, P=0.00021) and for treatment by time (F=1.90, P=0.0167). For treatment by time: At ZT19, almorexant at 100 mg/kg<vehicle. At ZT20, ZOL<almorexant at 30 mg/kg and vehicle. At ZT21, ZOL<all other conditions. At ZT22, vehicle<almorexant at 30 mg/kg. At ZT23, ZOL<almorexant at 10 mg/kg. At ZT24, almorexant at 10 mg/kg<vehicle. At ZT1, almorexant at all concentrations<ZOL. At ZT2, all other conditions<ZOL. At ZT3, all other conditions<ZOL. At ZT4, all other conditions<ZOL. At ZT5, all other conditions<ZOL. At ZT6, all other conditions<ZOL. At ZT7, all other conditions<ZOL. At ZT8, all other conditions<ZOL. At ZT9, all other conditions<ZOL. At ZT10, almorexant at 10 and 30 mg/kg and vehicle<ZOL. At ZT11, almorexant at 10 mg/kg and vehicle<ZOL; vehicle<almorexant at 100 mg/kg. At ZT12, almorexant at 10 and 30 mg/kg and vehicle<ZOL.

**Task 3.** *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.*
3a. Double-label immunohistochemistry with Fos and phenotypic markers (months 1 to 12).
3b. Assessment of hypnotic efficacy in saporin-lesioned rats (months 13 to 24).
3c. Assessment of hypnotic efficacy in transgenic mice (months 25 to 36).

Progress: In the absence of tissue from ALM- and ZOL-treated animals, there has been little experimental progress on this task in Year 1 and little expenditure of funds. The primary effort to date has been to order the appropriate antisera for these experiments and to initiate establishment of the immunohistochemical assays. We anticipate receiving the first group of 24 animals (8 ALM-treated, 8 ZOL-treated and 8 vehicle controls) by the end of August and work on this Task will accelerate at that time.

With the approval of Ms. Jennifer Shankle of MEDCOMM USAMRAA received on 10 Jun 2010, we re-budgeted some of the Year 1 funds to allow an upgrade of our existing Neurolucida and StereoInvestigator software from Microbrightfield, Inc. The PC and software upgrade was received in our laboratory on 26 Jul 2010. At the time of this writing, we are awaiting installation by a service representative. This data analysis package is necessary to conduct the cell counts necessary for quantification of the studies to be executed in Tasks 3 and 4a.

**Task 4.** *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*
4a. Effects of ALM and ZOL on sleep-active brain areas (months 1 to 12).
4b. BF adenosine (ADO) release in response to oral ALM and ZOL (months 1 to 24).
4c. BF adenosine (ADO) release in response to ALM and ZOL by dialysis (months 25 to 48).

Progress: As in Task 3, progress on experiments has been limited due to the construction issues described above. However, there has been tremendous progress on the infrastructure to support

Task 4. As indicated above, renovations are ongoing within the Animal Facility to construct a state-of-the-art 535 s.f. laboratory for EEG/EMG, behavioral performance and microdialysis sample collection.

In addition, shortly after funding of DR080789P1 (USAMRAA Grant W81XWH-09-2-0080), we were assigned a 985 square foot wet laboratory in which to establish an Analytical Neurochemistry Facility. LB212 has 6 bays and contains 6 fume hoods. To equip this laboratory, we relocated our one ESA CoulChem III High Performance Liquid Chromatograph (HPLC) used to detect dopamine and its metabolites to LB212 and, using SRI internal funds, we were able to acquire several other used ESA Coul Arrays from Roche Palo Alto. These additional machines enable us to measure acetylcholine, norepinephrine and its metabolites, and serotonin and its metabolites, as described in our 10 Feb 2010 email to Dr. del Carmen. In addition, an HPLC for the detection of adenosine, which had been requested in the original budget for DR080789P1 (USAMRAA Grant W81XWH-09-2-0080), was delivered on 19 Jul 2010. Lastly, on 10 Jun 2010, we received approval from Ms. Jennifer Shankle of MEDCOMM USAMRAA to re-budget some of the Year 1 funds to allow purchase of a 6$^{th}$ HPLC for determination of GABA, glutamate, glycine and other amino acids. We expect delivery of this machine by the end of this week. Thus, we will soon have capabilities well beyond the scope of the adenosine measurements proposed as Task 4b and 4c in the SOW.

We report here on progress to establish the functionality of these machines. Our ESA-Dionex service representative performed a full system reinstallation of all hardware equipment and software, and validated communication and automation capabilities for three ESA Coul Arrays. Service reports for each HPLC system are attached in Appendix 3. We then set up the three HPLCs for electrochemical detection, each to be optimized for specific neurotransmitter capabilities (System 1: norepinephrine, epinephrine, and dopamine; System 2: serotonin; System 3: acetylcholine). The last task was to validate internal standards tested specifically for each system to determine the lower limit of detection (i.e., what is the lowest level of neurotransmitter amount that can be measured *in vivo*).

For the data presented in Figure 5, individual samples were automatically injected by an autosampler into the HPLC/EC system (ESA-Dionex, Chelmsford, MA) for the generation of an external standard curve for norepinephrine (NE), epinephrine (Epi), and dopamine (DA). All neurotransmitter concentrations were made up as stock solutions and were dissolved in oxalic acid (1 mM, pH 3.6), and serially diluted to their final concentrations in 1 mM oxalic acid to preserve the stability of the samples. The mobile phase consisted of 150 mM $Na_2HPO_4$ (pH = 5.6), 3 mM sodium dodecyl sulfate, 50 mM EDTA, 10% methanol, and 15% acetylnitrile. NE, Epi, and DA were carried through with mobile phase, separated through an analytical MD-150x3.2-mm reversed phase column from ESA-Dionex, and oxidized/reduced using a Coul Array detector from ESA, Inc. Two electrodes were used, a reduction analytical electrode (E1, -0.1 V), and an oxidation analytical electrode (E2, 0.25 V). The area under the curve of each peak was measured using CoulArray Data Station 3.0 software (ESA, Inc.). Figure 5A shows the plot generated by the software and corresponding external standard data points for each neurotransmitter type: NE (blue circles), Epi (red circles), and DA (green circles). Individual samples of known concentrations were run in duplicate and averaged peak areas were integrated into a linear fit model to provide the goodness of fit (r value) for each neurotransmitter (NE, r=0.9995; Epi, r=0.9999, and DA, r=1.000). Figure 5B shows the calibration levels that were generated for each neurotransmitter and their corresponding averaged peak areas for each concentration. The lowest amounts of neurotransmitters detected using this calibration curve for

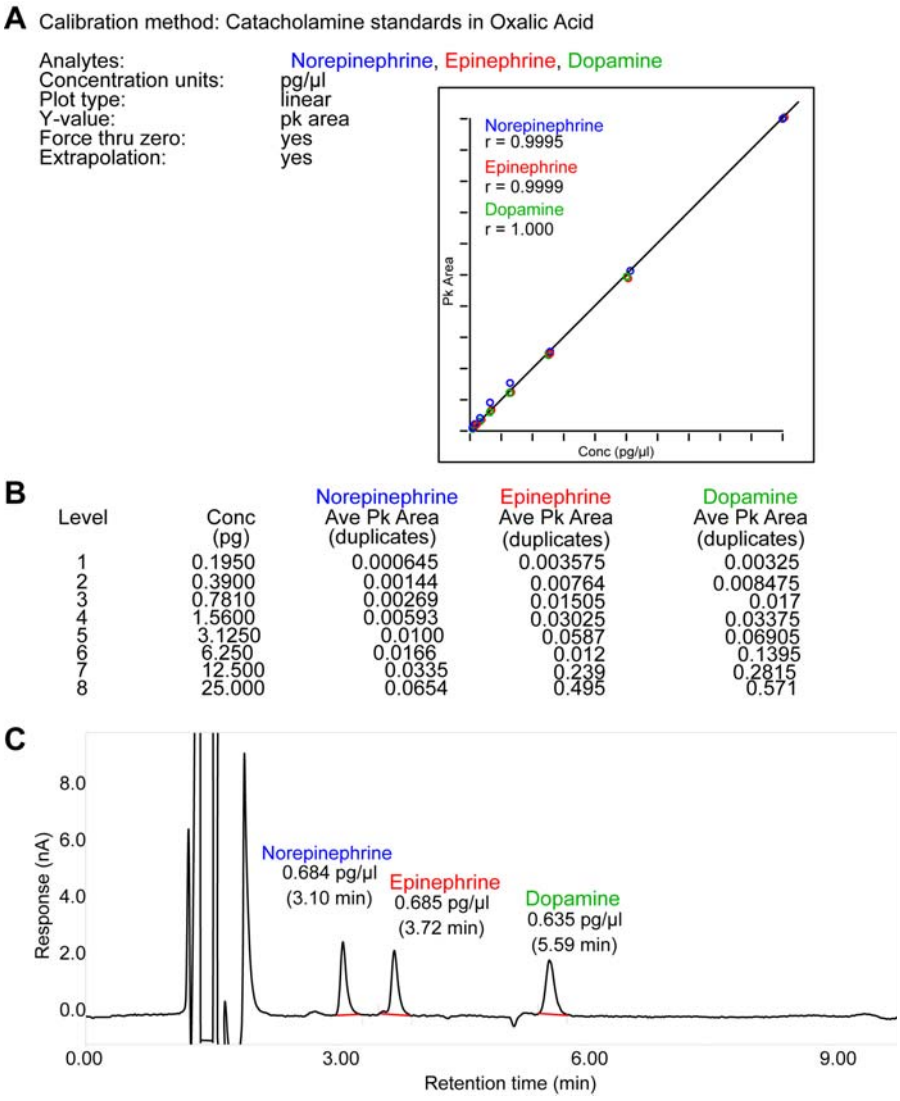



**Figure 5.** External standards, calibration, and HPLC/EC detection of norepinephrine (NE), epinephrine (Epi), and dopamine (DA)

NE, Epi, and DA were 200 fg, 200 fg, and 500 fg, respectively. Figure 5C depicts a chromatograph showing individual peaks for NE, Epi, and DA and their respective concentrations and retention times.

For the data presented in Figure 6, individual samples were injected by automation into the HPLC/EC system (ESA-Dionex) for the generation of an external standard curve for serotonin (5-HT). Serotonin concentrations was made up as a stock solution and was dissolved in oxalic acid (1 mM, pH 3.6), and serially diluted to final concentrations in 1 mM oxalic acid to preserve the stability of the samples. The mobile phase consisted of 150 mM $Na_2HPO_4$ (pH = 5.6), 3 mM sodium dodecyl sulfate, 50 mM EDTA, 10% methanol, and 15% acetylnitrile. 5-HT was carried through with mobile phase, separated through an analytical MD-150x3.2-mm reversed phase column from ESA-Dionex, and oxidized/reduced using a Coul Array detector from ESA, Inc. Two electrodes were used, a reduction analytical electrode (E1, -0.1 V), and an oxidation analytical electrode (E2, 0.25 V). The area under the curve of each peak was measured using CoulArray Data Station 3.0 software (ESA, Inc.). Figure 6A plots the peak area detected against the corresponding 5-HT external standards (blue circles). Individual samples of known concentrations were run in duplicate and averaged peak areas were integrated into a linear fit model to provide the goodness of fit (r value) for 5-HT (r=0.9968). Figure 6B shows the calibration levels that were generated for 5-HT and the corresponding averaged peak areas for each calibrated amount of 5-HT concentration. The lowest amount of neurotransmitter detection using this calibration curve for serotonin was 1 pg on column. Figure 6C presents a chromatograph showing an individual serotonin peak with its respective concentration and retention time.

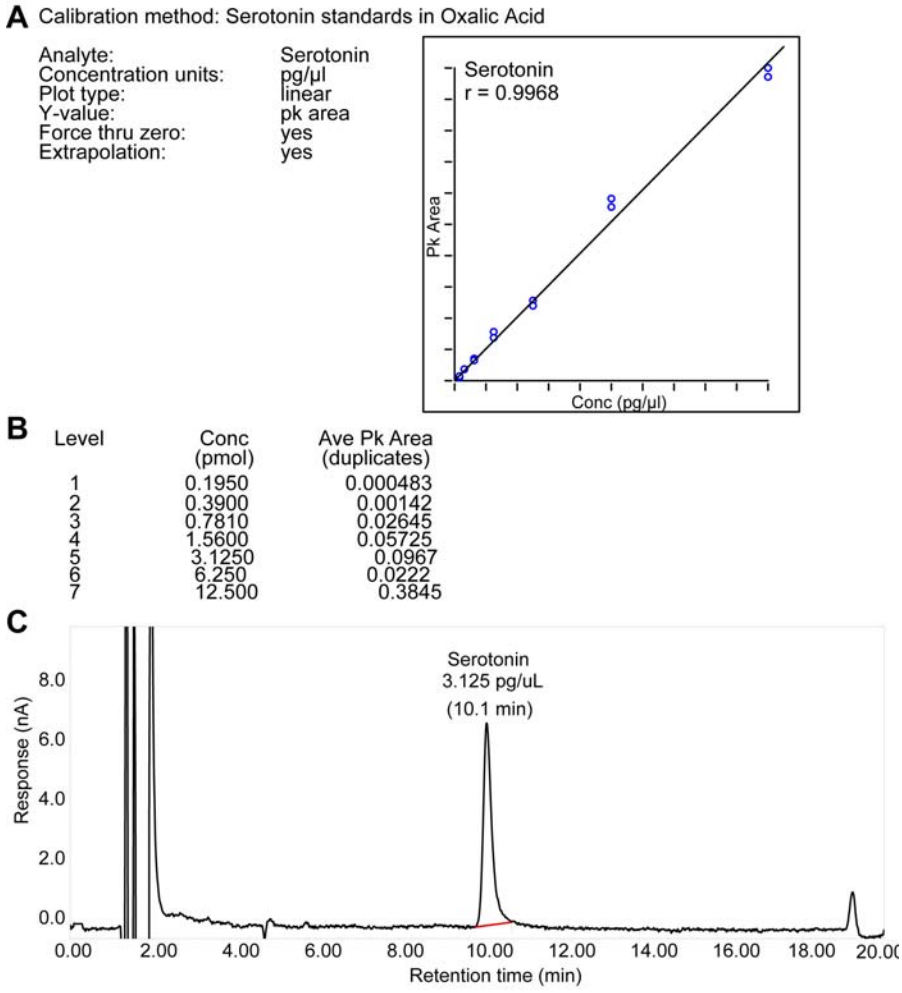



**Figure 6.** External standards, calibration, and HPLC/EC detection of serotonin (5-HT)

For the data presented in Figure 7, individual samples were automatically injected via an autosampler into the HPLC/EC system (ESA-Dionex) to generate an external standard curve for acetylcholine (ACh). ACh was made up as a stock solution and was dissolved in oxalic acid (1 mM, pH 3.6), and serially diluted to final concentrations in 1 mM oxalic acid to preserve the stability of the samples. The mobile phase consisted of 100 mM $Na_2HPO_4$ 2 mM 1-octanesulfonic acid, and adjusted to pH = 8.0 with phosphoric acid. This HPLC method uses a polymeric stationary phase to resolve choline (Ch) from ACh and is attached to an ACH-250x3.0-mm column. Analytes are then converted to hydrogen peroxide ($H_2O_2$) by a solid-phase reactor (containing immobilized choline oxidase and acetylcholinesterase enzymes). An additional enzyme reactor is attached to the column to eliminate the choline peak and avoid interference with ACh retention time. The $H_2O_2$ is detected amperometrically and quantified on a platinum (Pt) working electrode set to +300 mV with a solid-state palladium reference electrode. The area under the curve of each peak was measured using CoulArray Data Station 3.0 software (ESA, Inc.). Figure 7A plots peak areas detected against external ACh standards (blue circles). Individual samples of known concentrations were run in duplicate and averaged peak areas were integrated into a linear fit model to provide the goodness of fit (r value) for ACh (r=0.9980). Figure 7B lists the calibration levels that were generated for ACh and the corresponding averaged peak areas for each calibrated amount of ACh concentration. The lowest amount of neurotransmitter detection using this calibration curve for ACh was 200 fmol. Figure 7C depicts a chromatograph showing an individual ACh peak with its respective concentration and retention time.

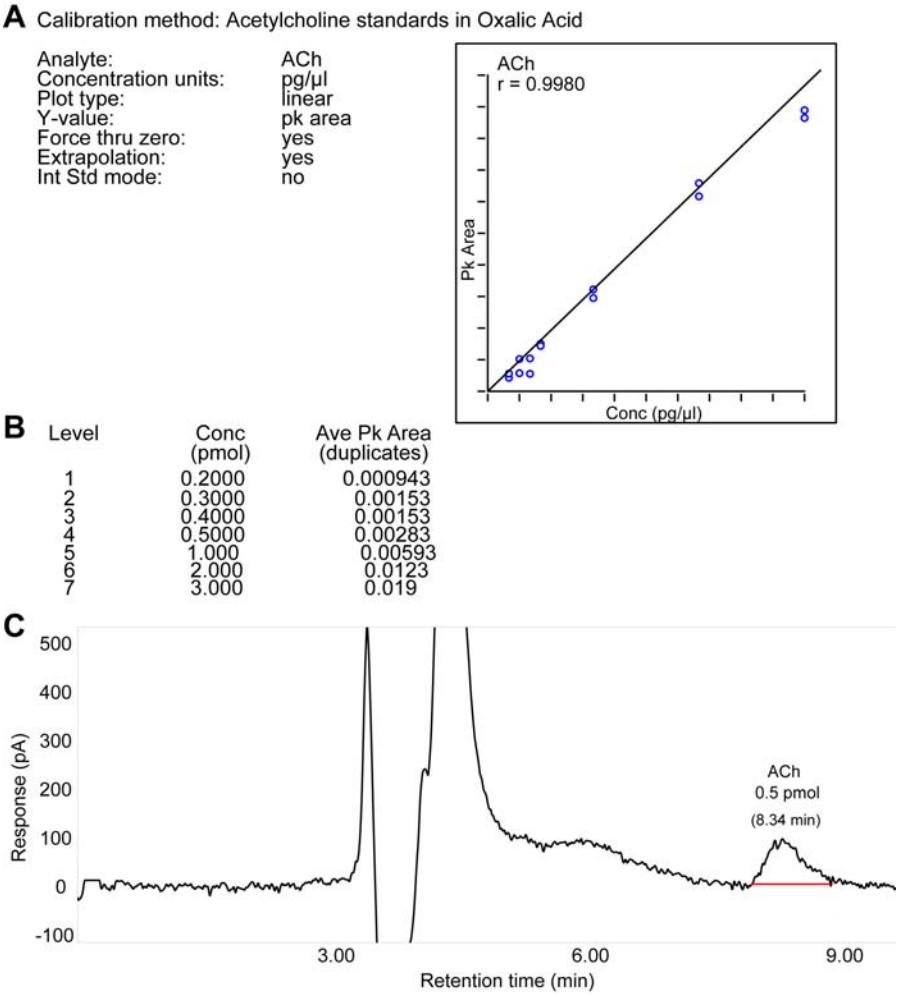



**Figure 7.** External standards, calibration, and HPLC/EC detection of acetylcholine (ACh).

**Task 5:** *Test the hypothesis that neural gene expression that occurs ALM-induced sleep more closely resembles that of spontaneous sleep than does ZOL-induced sleep.*
5a. Comparison of ALM and ZOL effects on expression of plasticity-related genes (months 37 to 48).
5b. Comparison of ALM and ZOL effects on brain gene expression in comparison to spontaneous sleep (months 37 to 48).

Progress: None anticipated prior to Year 3.

## KEY RESEARCH ACCOMPLISHMENTS

• Obtaining approval and commitment from SRI International to construct a new 535 s.f. laboratory suite within the Animal Facility to support the *in vivo* portion of this research program (construction initiated 19 May 2010; expected occupancy 3 Aug 2010).

• Set up of the water maze and video tracking system and initiation of data collection in a temporary location until construction of above-mentioned laboratory suite is completed

• Establishment of a 985 s.f. Analytical Neurochemistry Facility in LB212 to support this research program containing:

- 1 ESA CoulChem HPLC for analysis of dopamine and its metabolites relocated to LB212.
- 3 ESA Coul Array HPLCs for analysis of acetylcholine, norepinephrine and serotonin purchased from Roche Palo Alto on internal SRI funds; setup of machines supported by rebudgeting of current grant.
- 1 HPLC for analysis of adenosine received on 19 Jul 2010; awaiting installation.
- 1 HPLC for analysis of GABA, glutamate, glycine and other amino acids ordered on 14 Jun 2010; delivery expected this week.

• Full system reinstallation of all hardware equipment and software, and validated communication and automation capabilities for three ESA Coul Array HPLCs.

• Establishment of limits of detection for 4 of the ESA Coul Array HPLCs (Figs. 5-7).

• Determination of the effect of 3 doses of ALM vs. ZOL on sleep/wake and other physiological parameters in the Sprague-Dawley rat (Figs. 1-4)

• Upgrade of our existing Neurolucida and StereoInvestigator software from Microbrightfield, Inc. to facilitate cell counts necessary for quantification of the studies to be executed in Tasks 3 and 4a.

## REPORTABLE OUTCOMES

Manuscript in preparation:

Morairty SR, F.G. Revel, P. Malherbe, J-L. Moreau, K. Silveira, D. Valladao, J.G. Wettstein, T.S. Kilduff, E. Borroni. Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone.

## CONCLUSION

Preclinical data indicate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. Our research in this area has just commenced in Year 1 of this project but we expect to be able to address the validity of these claims and the mechanisms that may underlie them in the near future.

## REFERENCES

Morairty, S. L. Hedley, J. Flores, R. Martin and T. S. Kilduff (2008). Selective $5HT_{2A}$ and $5HT_6$ receptor antagonists promote sleep in rats. *Sleep* 31:34-44.

## APPENDICES

Appendix 1. Schematic floorplan of new EEG/EMG, Performance and Microdialysis Laboratory in LW103/105.

Appendix 2. Certificate of Analysis for synthesis of Almorexant by SRI International Medicinal Chemistry Laboratory.

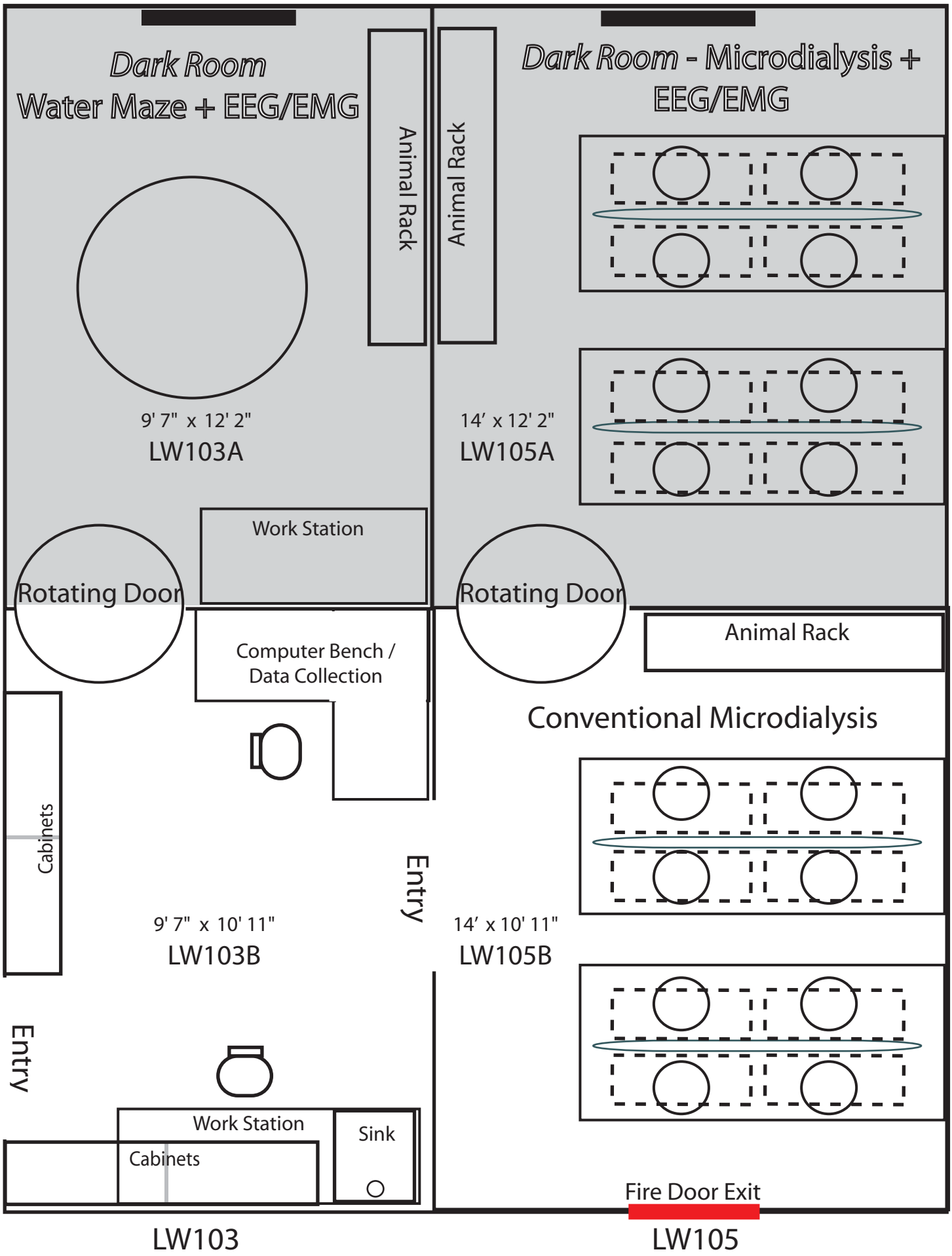
Appendix 3. Service report for the reinstallation and calibration of HPLC/EC system #1 (detection of norepinephrine, epinephrine, and dopamine) by ESA-Dionex, Inc.

Appendix 4. Service report for the reinstallation and calibration of HPLC/EC system system #2 (detection of serotonin) by ESA-Dionex, Inc.

Appendix 5. Service report for the reinstallation and calibration of HPLC/EC system #3 (detection of acetylcholine) by ESA-Dionex, Inc.

## Appendix 1

Schematic floorplan of new EEG/EMG,
Performance and Microdialysis Laboratory in LW103/105.


Dark Room
Water Maze + EEG/EMG
Animal Rack
Animal Rack
Dark Room - Microdialysis + EEG/EMG
9' 7" x 12' 2"
LW103A
14' x 12' 2"
LW105A
Work Station
Rotating Door
Rotating Door
Animal Rack
Computer Bench / Data Collection
Conventional Microdialysis
Cabinets
Entry
9' 7" x 10' 11"
LW103B
14' x 10' 11"
LW105B
Entry
Work Station
Sink
Cabinets
Fire Door Exit
LW103
LW105

## Appendix 2

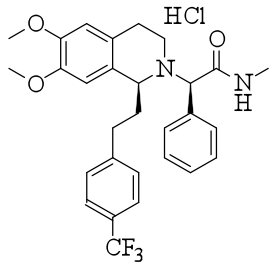
Certificate of Analysis for synthesis of Almorexant
by SRI International Medicinal Chemistry Laboratory.

# Certificate of Analysis

| | |
|---|---|
| **To:** Dr. Thomas Kilduff | **Date: 3/31/2010** |
| | **Contractor:** SRI International<br>333 Ravenswood Avenue<br>Menlo Park, CA 94025 |
| Almorexant (codenamed ACT-078573) | **P.I.:** Dr. Ling Jong |
| **Attn:** Stephen Morairty | **Project No:** P 19160.102 |

**Chemical Name:** (R)-2-((S)-6,7-dimethoxy-1-(4-(trifluoromethyl)phenethyl)-3,4-dihydroisoquinolin-2(1H)-yl)-N-methyl-2-phenylacetamide hydrochloride

**Structure:**




| **Lot #:** GL-S14124-24-F1 | | **ELEMENTAL** | **Calculated** | **Found** |
|---|---|---|---|---|
| **Formula:** C29H32ClF3N2O3 | | **C** | 63.44 | 63.41 |
| **Molecular Weight:** 549.02 | | **H** | 5.87 | 5.79 |
| **Date Synthesized:** 03/09/2010 | | **N** | 5.10 | 5.07 |
| **Purity:** > 99% | **MP:** 205-206 | | | |
| **Analyst:** Adria Lombardo | | | Amount: 26.05 g | |
| **Reviewer:** Dr. Gaoquan Li | | | Store in 12 oz brown bottle | |

**NMR:** $^1$H (300 MHz) ($CDCl_3$) δ 12.61 (br.s, 1 H), 9.53 (br.s, 1 H), 7.62 (br.s, 2 H), 7.48-7.30 (m, 5 H), 7.09 (d, J = 7.6 Hz, 2 H), 6.68 (s, 1 H), 5.73 (s, 1 H), 4.54 (d, J = 10.4 Hz, 1 H), 4.01-3.86 (m, 4 H), 3.86-3.76 (m, 2 H), 3.67 (s, 3 H), 3.34-3.22 (m, 1 H), 3.17-3.02 (m, 2 H), 2.89 (d, J = 4.4 Hz, 3 H), 2.86-2.73 (m, 2 H), 2.03-1.91 (m, 1 H)

**MS:** ESI+: 513.1, ESI-: 511.2 (non-salt Almorexant)

**UV:** (Methanol) $\lambda_{max}$ 286.1 nm (ε 4,099); 204.1 (ε 55,555)

**FTIR:** (Film) 3177.7, 3043.6, 2932.8, 2581.4, 1681.4, 1522.9, 1461.5, 1329.2, 1264.4, 1232.7, 1160.9, 1110.4, 1071.4, 1019.0, 743.9, 697.7 $cm^{-1}$

**TLC:** Analtech silica gel plates; 60% ethyl acetate/40% hexane $R_f$ = 0.25 (non-salt Almorexant)

**HPLC:** Phenomenex RP-C18; flow 1 mL/min; detection 284.0 nm; solvent 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B), 10%-90% B over 15 min; retention time 12.54 min; purity > 99 %

## Appendix 3

Service report for the reinstallation and calibration of HPLC/EC system #1 (detection of norepinephrine, epinephrine, and dopamine) by ESA-Dionex, Inc.

*ESA Biosciences*
*22 Alpha Road*
*Chelmsford, MA 01824*
*Telephone: (800) 275-0102*
*Fax: (978) 250-7092*

# *Service Report*

| | | | |
|---|---|---|---|
| Service Request # | 538304 | Engineer | Ralf Janssen |
| Type of Service | Installation | Date | February 26, 2010 |

## Customer Information

| | | | |
|---|---|---|---|
| *Company* | SRI International | | |
| *Address* | 333 Ravenswood Ave | | |
| *Address* | | | |
| *Address* | | | |
| *City* | Menlo Park | | |
| *State* | CA | *Zip* | 94025 |

## Instrument Information

| | | *Model* | *Serial Number* |
|---|---|---|---|
| *Detector* | CoulArray | 5600 | CA-856 |
| *Autosampler* | ESA | 542 | 60100 |
| *Pump 1* | ESA | 582 | S20104351055 |
| *Pump 2* | | | |
| *Other* | | | |

| | | | |
|---|---|---|---|
| Contact | Jacqueline Vasquez-DeRose | Customer Number | |
| Phone Number | 650-859-4794 | Purchase Order | 98-000219 |
| Fax Number | | Sales Order | 204584 |
| E-Mail Address | jacqueline.vasquez@sri.com | Work Order | |

**DESCRIPTION OF SERVICE TO BE PERFORMED**

Reinstall Coularray, Autosampler, Pump and computer. Install Coularray for Windows v3.1

**DESCRIPTION OF WORK PERFORMED**

Reinstallation of Coularray, Autosampler, Pump and computer completed. Installed Coularray for Windows v3.1

**Did the work performed on this request require that a product complaint be generated**

**If yes, Product complaint #**

**Service Request # 538304**

**PARTS REQUIRED**

| QUANTITY | PART NUMBER | DESCRIPTION | INVENTORY | LIST PRICE | DISCOUNT | TOTAL PRICE |
|---|---|---|---|---|---|---|
| 1 | 70-4003 | CoulArray for Windows v3.1 | SVS RALF | 2,860.00 | | 2,860.00 |
| | | | | | | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | PARTS SUBTOTAL | | 2,860.00 |
| | | | | DISCOUNT ON SUBTOTAL | | 2,860.00 |
| TRAVEL AND LABOR | | | | | | |
| 1 | 70-7645 | ESA HPLC Installation Service | | 1985 | | 1,985.00 |
| 1 | 70-7646 | ESA Software Installation Service | | 995 | | 995.00 |
| 1 | 70-7641 | Zone Travel Charge | | 400 | | 400.00 |
| | | | | | | - |
| | | | | TOTAL | $ | 6,240.00 |

**SUGGESTIONS / COMMENTS**

autosampler cooling tray liner deteriorating: recommend replacing.

**Service Representative**

***Ralf Janssen*** — *PLEASE PRINT*

*SIGNATURE* — *DATE* 2/26/2010

**Customer**

***Jacqueline Vazquez-DeRose*** — *PLEASE PRINT*

*SIGNATURE* — *DATE* 2/24/10

## Appendix 4

Service report for the reinstallation and calibration of
HPLC/EC system system #2 (detection of serotonin) by ESA-Dionex, Inc.

*ESA Biosciences*
*22 Alpha Road*
*Chelmsford, MA 01824*
*Telephone: (800) 275-0102*
*Fax: (978) 250-7092*

# *Service Report*

| | | | |
|---|---|---|---|
| Service Request # | 538306 | Engineer | Ralf Janssen |
| Type of Service | Installation | Date | February 26, 2010 |

## Customer Information

| | | | |
|---|---|---|---|
| *Company* | SRI International | | |
| *Address* | 333 Ravenswood Ave | | |
| *Address* | | | |
| *Address* | | | |
| *City* | Menlo Park | | |
| *State* | CA | *Zip* | 94025 |

## Instrument Information

| | | *Model* | *Serial Number* |
|---|---|---|---|
| *Detector* | CoulArray | 5600 | CA-909 |
| *Autosampler* | ESA | 542 | 50358 |
| *Pump 1* | ESA | 582 | S20104390994 |
| *Pump 2* | | | |
| *Other* | | | |

| | | | |
|---|---|---|---|
| Contact | Jacqueline Vazquez-DeRose+C106 | Customer Number | |
| Phone Number | 650-859-4794 | Purchase Order | 98-000219 |
| Fax Number | | Sales Order | 204584 |
| E-Mail Address | jacqueline.vasquez@sri.com | Work Order | |

**DESCRIPTION OF SERVICE TO BE PERFORMED**

Reinstall Coularray, Autosampler, Pump and computer. Install Coularray for Windows v3.1

**DESCRIPTION OF WORK PERFORMED**

Reinstallation of Coularray, Autosampler, Pump and computer completed. Installed Coularray for Windows v3.1

**Did the work performed on this request require that a product complaint be generated**

**If yes, Product complaint #**

**Service Request # 538306**

**PARTS REQUIRED**

| QUANTITY | PART NUMBER | DESCRIPTION | INVENTORY | LIST PRICE | DISCOUNT | TOTAL PRICE |
|---|---|---|---|---|---|---|
| 1 | 70-4003 | CoulArray for Windows v3.1 | SVS RALF | 2,860.00 | | 2,860.00 |
| | | | | | | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | PARTS SUBTOTAL | | 2,860.00 |
| | | | | DISCOUNT ON SUBTOTAL | | 2,860.00 |
| TRAVEL AND LABOR | | | | | | |
| 1 | 70-7645 | ESA HPLC Installation Service | | 1985 | | 1,985.00 |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | TOTAL | $ | 4,845.00 |

**SUGGESTIONS / COMMENTS**

**Service Representative**

***Ralf Janssen*** — *PLEASE PRINT*

*SIGNATURE* — *DATE* 2/26/2010

**Customer**

***Jacqueline Vazquez-DeRose*** — *PLEASE PRINT*

*SIGNATURE* — *DATE* 2/26/10

## Appendix 5

Service report for the reinstallation and calibration of HPLC/EC system #3 (detection of acetylcholine) by ESA-Dionex, Inc.

*ESA Biosciences*
*22 Alpha Road*
*Chelmsford, MA 01824*
*Telephone: (800) 275-0102*
*Fax: (978) 250-7092*

# *Service Report*

| | | | |
|---|---|---|---|
| Service Request # | 538308 | Engineer | Ralf Janssen |
| Type of Service | Installation | Date | February 26, 2010 |

## Customer Information

| | |
|---|---|
| *Company* | SRI International |
| *Address* | 333 Ravenswood Ave |
| *Address* | |
| *Address* | |
| *City* | Menlo Park |
| *State* | CA *Zip* 94025 |

## Instrument Information

| | | *Model* | *Serial Number* |
|---|---|---|---|
| *Detector* | CoulArray | 5600 | CA-935 |
| *Autosampler* | ESA | 542 | 40266 |
| *Pump 1* | ESA | 582 | S20104250866 |
| *Pump 2* | | | |
| *Other* | | | |

| | | | |
|---|---|---|---|
| Contact | Jacqueline Vazquez-DeRose | Customer Number | |
| Phone Number | 650-859-4794 | Purchase Order | 98-000219 |
| Fax Number | | Sales Order | 204584 |
| E-Mail Address | jacqueline.vasquez@sri.com | Work Order | |

**DESCRIPTION OF SERVICE TO BE PERFORMED**

Reinstall Coularray, Autosampler, Pump and computer. Install Coularray for Windows v3.1

**DESCRIPTION OF WORK PERFORMED**

Reinstallation of Coularray, Autosampler, Pump and computer completed. Installed Coularray for Windows v3.1

**Did the work performed on this request require that a product complaint be generated**

**If yes, Product complaint #**

**Service Request # 538308**

**PARTS REQUIRED**

| QUANTITY | PART NUMBER | DESCRIPTION | INVENTORY | LIST PRICE | DISCOUNT | TOTAL PRICE |
|---|---|---|---|---|---|---|
| 1 | 70-4003 | CoulArray for Windows v3.1 | SVS RALF | 2,860.00 | | 2,860.00 |
| | | | | | | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | PARTS SUBTOTAL | | 2,860.00 |
| | | | | DISCOUNT ON SUBTOTAL | | 2,860.00 |
| TRAVEL AND LABOR | | | | | | |
| 1 | 70-7645 | ESA HPLC Installation Service | | 1985 | | 1,985.00 |
| | | | | | | - |
| | | | | | | - |
| | | | | | | - |
| | | | | TOTAL | $ | 4,845.00 |

**SUGGESTIONS / COMMENTS**

**Service Representative**

***Ralf Janssen*** — *PLEASE PRINT*

*SIGNATURE* — *DATE* 2/26/2010

**Customer**

***Jacqueline Vazquez-DeRose*** — *PLEASE PRINT*

*SIGNATURE* — *DATE* 2/26/10